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Original Article

PREVALENCE AND PROGNOSIS OF MICROSATELLITE INSTABILITY IN OESOGASTRIC ADENOCARCINOMA, NORDICAP 16-01.

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TA: study conception and design and manuscript critical revision.

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Abstract

Background

The prevalence and prognosis association of microsatellite instability (MSI) in oesogastric junction and gastric adenocarcinoma (OGC) have been reported with conflicting results.

Methods

Patients with OGC from 2010 to 2015 were enrolled in this retrospective multicenter study. MSI was determined by genotyping. *MLH1* promoter methylation and *BRAF V600E* mutation were screened in the MSI tumors.

Results

Among 315 tumors analyzed, 39 (12.4%) were of the MSI phenotype. Compared to MSS tumors, MSI tumors were more frequent in patients ≥ 70 years (17% vs 9%, $p=0.048$) and in gastric antral primary (20% versus 5% in junction tumor and 12% in fundus tumor. Among 29 MSI tumors analyzed, 28 had a loss of MLH1 protein expression and 27 had *MLH1* promoter hypermethylation. None had a *BRAF V600E* mutation. The 4-year cumulative incidence of recurrence for patients with resected tumour

was significantly lower in dMMR tumors versus pMMR tumors (17% versus 47%, $p=0.01$). For the patients with unresectable tumor the median overall survival was 11 months in MSS group and 14 months in MSI group ($p=0.24$).

Conclusion

MSI prevalence in OGC was 12.4%, associated with antral localization and advanced age. Patients with MSI tumors had a lower cumulative incidence of recurrence after surgery. MSI phenotype was mainly associated with loss of *MLH1* protein expression, *MLH1* promotor hypermethylation and had no *BRAF V600E* mutation.

Key words : gastric cancer, mismatch repair deficiency, microsatellite instability

BACKGROUND

Oesogastric junction and gastric adenocarcinoma (OGC) are one of the most frequent cancers worldwide and despite the decreasing incidence and mortality rates, OGC is the third most common cancer-related cause of death (1). Prognosis of OGC is heterogeneous from 59% at 5 years for localized tumor to less than 2% for metastatic cancers. Current standard treatment for resectable OGC (stage II-III) includes peri-operative chemotherapy. This is only based on preoperative radiological staging with no validated biomarkers. However, relapse is observed in more than half of the patients. A better tumor molecular characterization is needed to adapt the treatment for both localized and metastatic cancer.

A molecular classification had been proposed in 2014 based on the study of 295 OGC (2) which separated gastric cancers into 4 groups (tumors positive for Epstein–Barr virus, unstable microsatellite tumors, stable genomic tumors, and tumors with chromosomal instability). Microsatellite instability (MSI) was assessed using four mononucleotide and three dinucleotide repeat loci testing in the latter study and a prevalence of 22% was reported for MSI.

MSI testing or mismatch repair deficiency (dMMR) assessed by immunohistochemistry is routinely performed in colorectal cancer (CRC) and found to be associated to older patients (3) , female patients, proximal tumors and less differentiated tumors (4,5). MSI has a prognostic value in CRC: positive prognostic factor for early stages (stage II/III) (6) and negative prognostic factor in metastatic CRC (7).

In OGC MSI studies, a prevalence from 8 to 23% has been reported (8–14). Such tumors seemed to be associated with female sex, older patients, intestinal Lauren histological type, distal location, lack of lymph node invasion or distant metastases (15). These studies have assessed the prognostic association of MSI or dMMR phenotypes, showing a better survival. However, these studies have a small number of patients and there is a discordance between Europe and Asia.

Moreover, two studies derived from prospective clinical trials have suggested, the lack of efficacy of post-operative or peri-operative chemotherapy in patients with dMMR OGC (14,16). A better overall survival (OS) was observed in patients with dMMR OGC treated with surgery alone compare to those treated by peri-operative chemotherapy in the MAGIC *post-hoc* analysis (14). In *post-hoc* analysis of CLASSIC trial (16), MSI status is predicting benefit from adjuvant chemotherapy after gastrectomy in stage II/III gastric cancer. Nevertheless, in this cohort there were few older patients.

In consequence caution has been recommended for peri-operative or adjuvant chemotherapy in case of MSI/dMMR OGC in recent guidelines (17).

The aim of our study was to evaluate the prevalence of MSI in an unselected population of OGC and to define clinical and histological features and prognosis associated to MSI OGC. Moreover we

assessed the MMR protein expression by immunochemistry in MSI tumors, and screened MSI OGCs for *BRAF* mutation and *MLH1* promoter methylation.

PATIENTS AND METHODS

Study population:

We have performed a multicenter retrospective study, which has enrolled all consecutive patients from 2010 to 2015 with OGC treated in 5 French centers (NORDICAP network), for which tumor samples (biopsy or surgical samples) and clinical data with at least one-year follow up were available. The clinical and follow-up data were retrospectively collected and include demographics data, cancer stage, lymph node invasion, treatment history, recurrence and survival data. For all patients included, tumor samples were reviewed by a senior pathologist (CG, NP, RK or NC) to confirm histological features.

MSI and MMR assessment:

Normal mucosa and tumor area with >30% of tumor cells were selected from paraffin embedded specimens by a senior pathologist. DNA was extracted from a selected area using the Maxwell® 16 FFPE Plus LEV DNA Purification Kit on a Maxwell 16 System (Promega Corporation, WI, USA). The MSI status was determined either according to Buhard *et al*(18) or by using the Promega panel (MSI Analysis System, Version 1.2, Promega). Both methods included 5 mononucleotide repeat markers (BAT-25, BAT-26, NR-21, NR-24 and MONO-27). Analyses were conducted out according to manufacturers' recommendations. MSI was defined by two or more markers with instability. If none or only one marker showed instability, the tumor was considered microsatellite stable (MSS). *BRAF V600E* mutation screening was also performed in MSI tumors.

For tumors identified as MSI by molecular analysis (or in the cases of undetermined MSI status), MMR protein expression was assessed by immunochemistry (IHC) using an automated immunohistochemical stainer (streptavidin-peroxidase protocol, Bond III, Leica). Serial sections were incubated with the following antibodies: MLH1 (dilution 1:25, Agilent, clone ES05), PMS2 (ready to use, Agilent, clone EP51), MSH2 (ready to use, Leica, clone 79H11) and MSH6 (dilution1:50, Agilent, clone EP49). Loss of MMR protein expression was considered in case of complete absence of nuclear staining of neoplastic cells. Lymphocytes were used as internal positive control.

BRAF V600E status

BRAF V600E mutation screening was performed only in tumors with MSI. Codon 600 of *BRAF* was analyzed either: by real-time PCR using the *BRAF v600* mutation detection kit (AmoyDx) on a LC 480

thermocycler (Roche Diagnostics, Meylan, France), or by pyrosequencing using the Therascreen BRAF pyrokit on a PyroMark Q24 system (Qiagen, Courtaboeuf, France). Analyses were carried out according to manufacturers' recommendations.

MLH1 promotor methylation

MLH1 promotor methylation was determined by the MethyLight method, described by Park et al (19). This method is based on PCR with primer pairs specific for a more proximal region of the *MLH1* promoter that amplify methylated DNA after bisulfite treatment, together with a fluorescent probe that is also specific for the methylated sequence, thus allowing quantitative measurement of the proportion of methylated DNA as compared to controls.

Statistical analysis

Variables are described as count and proportion for qualitative variables and median and interquartile range for quantitative variables. Comparison between patients with MSI tumors versus MSS tumors were performed using Wilcoxon rank sum test or Fisher exact test, depending on the nature of the variable

OS was defined as the time between diagnosis and death. Recurrence-free survival (RFS) was defined in the subgroup of patients who received surgery as the time between surgery and first recurrence or death. In patients with synchronous metastases or unresectable tumor, progression-free survival (PFS) was defined as the time between diagnosis and first progression or death. OS, RFS and PFS were estimated using the Kaplan–Meier method and compared between groups by log rank test. In patients that have a tumor resection, the cumulative incidence of recurrence was estimated considering death without recurrence as a competing event. Effect of MSI status on risk of death, risk of recurrence or death, and risk of progression or death was estimated using Hazard Ratio (HR) by fitting Cox proportional hazard models. Cumulative incidence of recurrence was estimated and compared according to MSI status by Gray test, and effect of MSI status on cause-specific risk of recurrence was estimated using cause-specific Hazard Ratio (csHR).

All analyses were conducted using R v. 3.5.0 and all test were two-sided, with $p < 0.05$ considered as significant.

RESULTS

Prevalence of MSI phenotype

Three hundred and eighty-eight patients were treated for OGC between January 2010 and December 2015 in our network of five university hospital. Seventy-three patients were excluded: 40 patients

because there were no sample available for analyses, 32 patients because the material was unsuitable for molecular analysis, and 1 patient, because there was no residual tumor tissue and no pre-operative sample. Finally, 315 patients were included in the study. In 302 patients the MSI analysis could be performed and validated, whereas in 13 patients the molecular analysis was inconclusive. In these 13 patients an IHC screening was performed (figure 1). MSI analysis was performed on surgical samples for 188 patients among them 104 were previously treated by neo-adjuvant chemotherapy and on biopsies samples for 125 patients. For two patients it was studied on both biopsies and surgical samples with the same result.

Thirty-nine patients (12.4%) had a MSI tumor determined by molecular analysis, 263 patients (83.5%) had MSS tumor. In the 13 cases with an undetermined MSI status the screening by IHC revealed no loss of expression of a MMR protein and these cases were considered as proficient MSS.

Clinical characteristics according to MSI status

Patient and tumor characteristics are reported in table 1. Patients with MSI tumors were older than patients with MSS tumors. Furthermore, the prevalence of MSI tumor in patients ≥ 70 years old was twice the prevalence of MSI tumor in patients < 70 years (17% versus 9%, $p=0.048$). The prevalence of MSI tumors increased along the upper digestive tract, 5/99 (5%) in junction tumors, 9/73 (12%) in fundus tumors and 24/119 (20%) in antrum tumors. Worthy of note, the MSI phenotype was rare in *linitis plastica* 1/24 (4%). The prevalence of MSI was 8/89 (9%) in metastatic tumors and 31/174 (18%) in localized tumors ($p=0.34$). Surgery was performed in 77% of MSI tumors and 69% of MSS tumors ($p=0.36$). In the subgroup of 221 patients with resected tumor, there was a higher prevalence of MSI phenotype in tumors with no lymph node invasion (N0 tumors): 17/78 (22%) compared to tumors with lymph node metastasis 12/132 (9%) ($p=0.013$). Peri-operative chemotherapy was less frequently performed in patients with MSI tumor (33%) compared to patients with MSS tumor (57%), ($p=0.018$). None of the patients in any group had immunotherapy. R0 surgery was obtained in 93% of MSI group treated by surgery and 88% of MSS group ($p=0.54$).

Concordance of molecular analysis and IHC:

Among the 39 MSI tumors identified by molecular analysis, 29 had MMR protein expression analyzed by IHC. Twenty-eight tumors showed loss of expression of MLH1: one with isolated MLH1 loss of expression, and 27 associated with PMS2 protein loss of expression. One tumor showed MSH6 protein loss of expression.

BRAF mutation and MLH1 promoter methylation

BRAF V600E mutation was assessed in 37 of the 39 patients with MSI tumor, and no mutation was found. Among the 28 tumors with loss of MLH1 expression, *MLH1* promoter methylation was determined. All of them except one had hypermethylation of the *MLH1* promoter and were thus classified as likely sporadic. The tumors that did not present *MLH1* promoter hypermethylation had no identified Lynch syndrome or history of familial cancer.

Recurrence and survival analysis

The median follow-up was 39 months. In the whole study population, there was no difference in median OS according to MSI/MSS status: 27 months for MSI tumors compared to 30 months for MSS tumors (HR=0.88, 95% CI 0.53-1.4, p=0.63).

In the 221 patients with resected tumors, the median RFS was not reached for patients with MSI tumor and was 22 months for patients with MSS tumor (HR=0.69, 95% CI 0.38–1.26, p=0.23). At 2 years, the cumulative recurrence rate was 17% in MSI group versus 39% in MSS group. At 4 years the cumulative recurrence rate was stable at 17% in MSI group and 47% in MSS group (p=0.013) (figure 2). There was no effect of peri-operative chemotherapy on RFS between patients with MSI or MSS phenotype (p>0.05). In a multivariate analysis, in patients with resected tumor who were not metastatic at diagnosis (N=199), MSS status was not a prognostic factor of risk of recurrence or death. Two risk factors were identified : uncompleted resections (R1 or R2) and lymph node invasion (table 2).

In the 94 patients with synchronous metastases or unresected primary tumor, the median PFS was 8.2 months in MSI group and 6.7 months in MSS group (HR=0.75, 95% CI 0.41-1.37, p=0.35) and median OS was 14 months in MSI group and 11 months in MSS group (HR=0.57, 95% CI 0.23 – 1.43, p=0.24).

DISCUSSION

In this study, we found a prevalence of 12.4% of MSI phenotype in a large cohort of patients with OGC, which is consistent with other data found in the literature (12,14,20) and a recent meta-analysis (15). We also showed that MSI phenotypes were associated with older patients, distal gastric localization and less node invasion. The increased prevalence of MSI phenotype in gastric cancer according to age, is also consistent with what is observed in colorectal cancer (3).

We found a lower cumulative recurrence rate after R0 surgery for patients with MSI tumors compared to patients with MSS tumors but it is not significant in multivariate analysis in patients with localized primary tumors who had surgery (of note, 22 patients had surgery for metastatic disease and were excluded from this analysis). This may be explained by less node metastasis

associated with MSI tumors. It must be pointed out that in colon cancer the association between NO status and MSI is reported and that MSI is an independent prognostic factor (4). The lower recurrence rate was observed despite the fact that MSI tumor patients had received less neoadjuvant chemotherapy. This may be due to the older age of patients with MSI than MSS tumors rather than the node status poorly evaluated before the surgery. Some concerns have been raised about the effect of neo-adjuvant chemotherapy in patients with MSI tumors. Smyth and al found in a post hoc analysis of the MAGIC trial that patients with MSI tumors have a better OS compared with patients with MSS tumors when treated with surgery alone, whereas they have a shorter OS when treated with a peri-operative chemotherapy as well as surgery. They also reported that no patient with MSI tumor had a significant pathologic response (14). Moreover, a meta-analysis on individual data of four randomized trial, reports that the the patients with a resectable MSI gastric cancer had no survival benefit if treated by chemotherapy(21). At the opposite, Haag et al (22) showed that MSI phenotype is a favorable prognostic marker in gastric cancer patients undergoing neoadjuvant treatment despite a poor histologic response. However, these 2 studies included a very small number of patients (9 patients with MSI and neoadjuvant chemotherapy in both). Our results suggest that patients with MSI tumors did not experience a deleterious effect of neo-adjuvant chemotherapy but the low number of patients, the retrospective design and the stage heterogeneity between MSI and MSS tumors prevent any conclusion about peri-operative chemotherapy effect in MSI patient in our study . Regarding post-operative treatment, Choi and al in a post hoc analysis of the CLASSIC trial (16) and Kim and al (10), in a retrospective cohort including 105 patients with MSI tumor, show no benefit of adjuvant chemotherapy in these patients. The peri-operative chemotherapy impact in patients with MSI gastric cancer remains unclear. This point is essential since most of patients with MSI tumors are older patients with comorbidities and poor performance status leading to worst chemotherapy tolerance. Molecular classification should help determine patients who would benefit from peri-operative treatment. We need more studies to determine the prognosis impact of MSI phenotypes, especially prospective studies as in all the previous studies, MSI was assessed retrospectively. Moreover immunotherapy may be an alternative peri-operative treatment in patient with MSI tumour and this option is currently under evaluation (23).

Regarding mechanism leading to MSI phenotype, we found that 28 of 29 MSI tumors tested by IHC had loss of MLH1 expression. This is consistent with previous findings showing that, in oesogastric adenocarcinoma, the majority of MSI phenotype cases was related to loss of MLH1 expression (14,22). Moreover, we showed that loss of MLH1 expression was related to *MLH1* promotor methylation. Nevertheless, MSI phenotype was not associated with *BRAF* mutation. This is very different in sporadic colorectal cancer for which MSI phenotype is mainly associated with *BRAF V600E* mutation especially in older patients (3). A previous study reports 3% of prevalence of *BRAF*

D594G mutation, unrelated to MSI phenotype, in gastric cancer but no *BRAF V600E* mutation(24). The limitations of our study are the retrospective design, the small series of patients. But our study presents real life results in a European population. Another limitation of the study is that the IHC evaluation for MMR protein expression was not performed in all samples. Nevertheless it has been previously shown that there was 97.6% of concordance between the two methods in gastric cancer (14). Finally, the MSI testing was performed in 104 patients after neo-adjuvant chemotherapy that might have modified the result of the testing. Nevertheless, two patients in our study had both pre and post chemotherapy samples and were tested for MSI on both, and the MSI status result is the same for both samples. Moreover, we have preliminary data on 25 patients from another ongoing study showing no difference on MSI status determined by PCR before and after neo-adjuvant chemotherapy.

In conclusion, our study showed a high prevalence of MSI in distal gastric adenocarcinoma and older patients. MSI was mainly due to MLH1 promotor methylation without association with *BRAF* mutation. Patients with dMMR tumor experienced less recurrence. The predictive value of MSI phenotype for neo-adjuvant therapy efficacy remains controversial and deserves further evaluation.

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Table 1: patients and tumor characteristics

Variable (n)	Modality	MSI (n=39)	MSS (n=276)	p-value
Median age (309)		70.25 [62.45 - 79.1]	63.6 [52.85 - 73.4]	0.0084
Age (309)	<70 years	18 (47.4%)	176 (64.9%)	0.0478
	≥ 70 years	20 (52.6%)	95 (35.1%)	
Gender (315)	Female	18 (46.2%)	88 (31.9%)	0.1020
	Male	21 (53.8%)	188 (68.1%)	
Localization (315)	Junction	5 (12.8%)	94 (34.1%)	0.0041
	Fundus	9 (23.1%)	64 (23.2%)	
	Antrum	24 (61.5%)	95 (34.4%)	
	<i>Linitis plastica</i>	1 (2.6%)	23 (8.3%)	
Stage at diagnosis (313)	Localized	31 (79.5%)	193 (70.4%)	0.3420
	Metastatic	8 (20.5%)	81 (29.6%)	
Sub group of patient with resected tumor (n=221)		29 (74%)	192 (69%)	
Lauren classification (129)				
	Diffuse type	2 (11.1%)	36 (32.4%)	0.2190
	Intestinal type	9 (50%)	46 (41.4%)	
	Mixed type or unclassified	7 (38.9%)	29 (26.1%)	
Quality of resection (217)	R0	27 (93.1%)	165 (87.8%)	0.5420
	R1/R2	2 (6.9%)	23 (12.2%)	
TNM stage (203)	T1	4 (13.8%)	26 (13.7%)	0.674
	T2	8 (27.6%)	32 (16.8%)	
	T3	16 (55.2%)	92 (48.4%)	
	T4	1 (3.4%)	24 (12.6%)	
Lymph node status (209)	N0	17 (58.6%)	60 (33.5%)	0.0126
	N+	12 (41.4%)	119 (66.5%)	
Peri-operative Chemotherapy (219)	No	20 (66.7%)	81 (42.9%)	0.0180
	Yes	10 (33.3%)	108 (57.1%)	

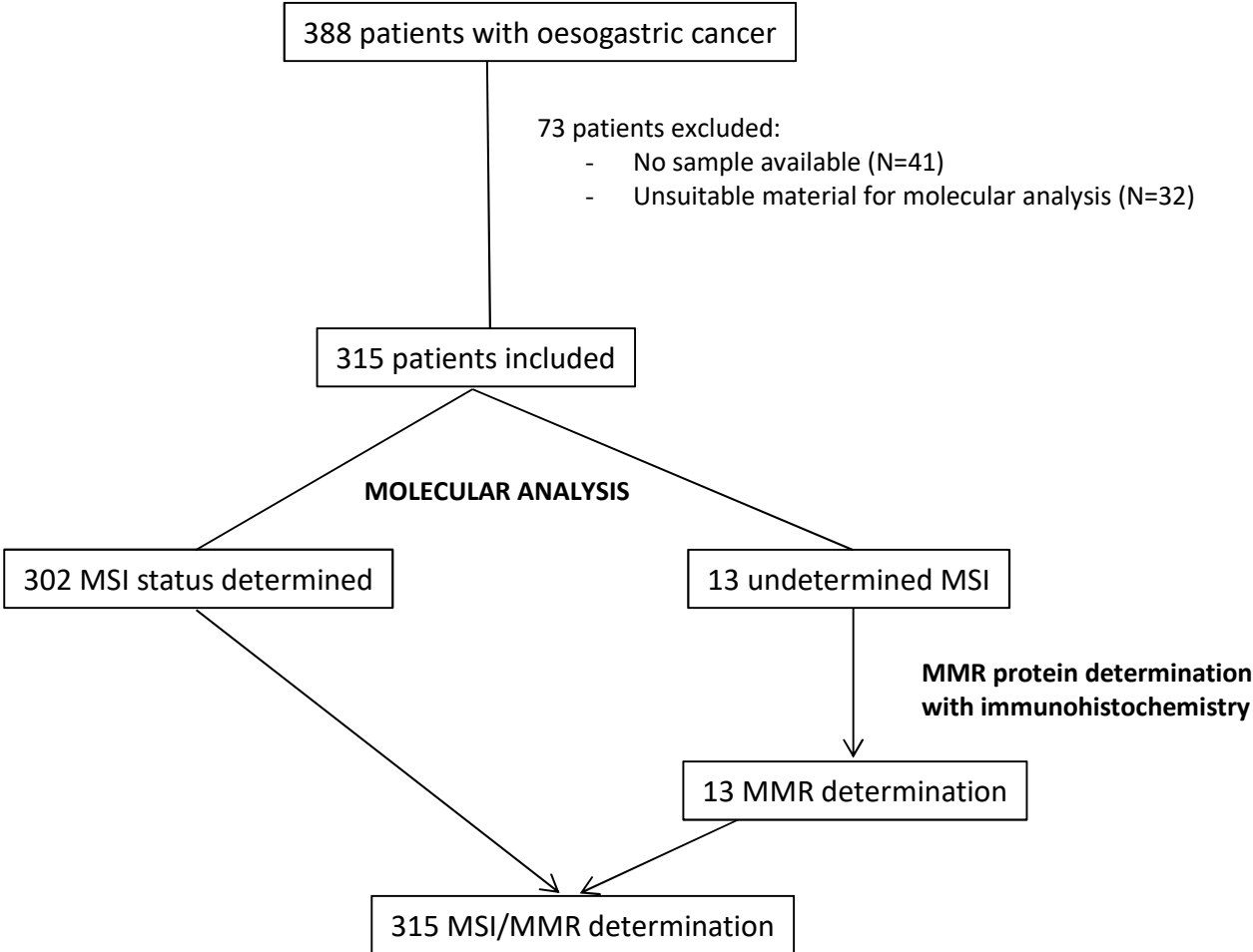
MSI: microsatellite instability

MSS: microsatellite stability

Table 2 multivariable analysis of prognostic factors of risk of recurrence among patients with resected tumor not metastatic at diagnosis (N=199)

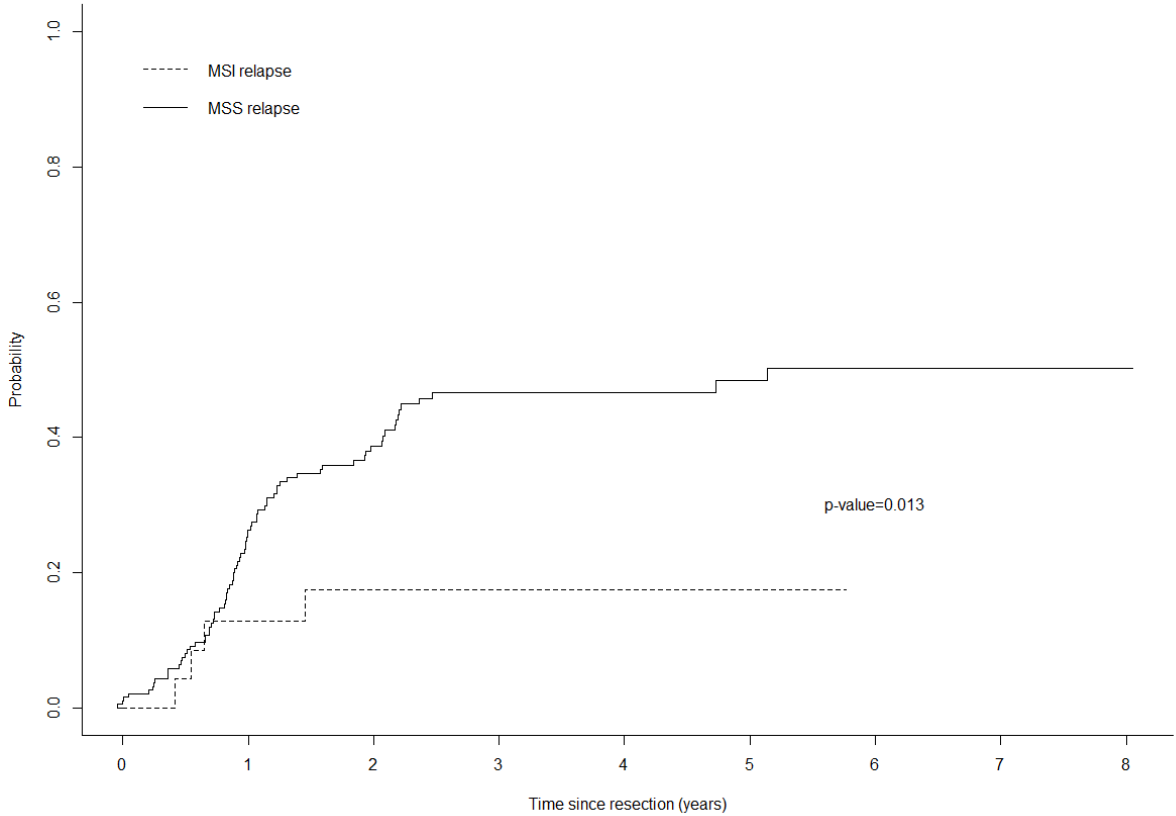
	csHR	95%CI	P-value
MSS	1.61	[0.57 – 4.53]	0.37
Age (for 1 year increase)	0.99	[0.97 – 1.01]	0.43
Peri-operative chemotherapy	1.95	[0.98 – 3.88]	0.056
R1/R2 resection	2.96	[1.53- 5.71]	0.0012
N+	2.89	[1.53 – 5.44]	0.0011

Figure 1: Flow chart of the study



MSI: microsatellite instability
MMR: mismatch repair

Figure 2: cumulative recurrence rate according to MSI status



MSI: microsatellite instability
MSS: microsatellite stability